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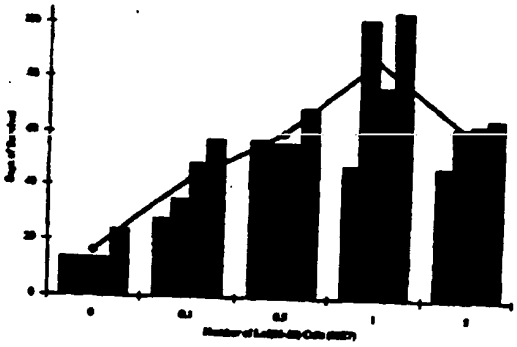
(54) Title: SYSTEM FOR THE MAINTENANCE, GROWTH AND DIFFERENTIATION OF HUMAN AND NON-HUMAN PRIMATE PLURIPOTENT STEM, PROGENITOR AND MATURE BONE MARROW CELLS

Survival of Lethally Irradiated SCID Mice Reconstituted with the Human Stromal Cell Line Lof(11-10)



Mice were irradiated with 400 rads. Four mice in each group were injected i.p. with the indicated number of cells.

(57) Abstract

The present invention relates to a method of engrafting human or non-human primate pluripotent stem cells, such as CD34+ cells, in a mouse. The method comprises administering to irradiated mice immortalized human or non-human primate bone marrow stromal cells which express cytokines supporting growth of cells, such as CD34+ cells, and, sequentially or simultaneously, administering purified human or non-human primate CD34+ cells. The stromal cells and CD34+ cells can be administered by intraperitoneal or intravenous injections. The invention can be used to evaluate gene therapy protocols for human CD34+ cells as well as having applications in the treatment of patients exposed to irradiation such as during a bone marrow transplant. The invention also provides an animal model for the study of human hematopoiesis.

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SYSTEM FOR THE MAINTENANCE, GROWTH AND DIFFERENTIATION OF HUMAN AND NON-HUMAN PRIMATE PLURIPOTENT STEM, PROGENITOR AND MATURE BONE MARROW CELLS

SPECIFICATION

Background of the Invention

Field of the Invention

The present invention relates to the use of an immortalized human cell line to create a human microenvironment in mice for the study of human disease states. In this human microenvironment, cells such as human and non-human primate pluripotent stem cells can be maintained and differentiated. In particular, the present invention relates to the injection of an immortalized human stromal cell line (such as Lof(11-10)) into mice to create a human microenvironment supplying human cytokines that are necessary to support the engraftment, maintenance and differentiation of human and non-human primate CD34+ stem cells. Such a human microenvironment provides a valuable animal model for the study of human hematopoiesis.

Description of the Prior Art

Biomedical research relies heavily on animal models to gain an understanding of the various disease states in human. Using animal models, researchers hope to gain an understanding of disease states which will enable them to intervene in the disease process. Animal models have been successfully used to study a range of human diseases, everything from the safety of aspirin and metal metabolism to bacterial and viral infections. For example, mice have been used to investigate Alzheimer's disease (Wi et al., Chung Kuo Chung Hsi I Chieh Ho Tsa Chih (China) 12:622, 1992), staphylococcal infection (Bel'skii et al., Zh. Mikrobiol Epidemiol. Immunobiol. (USSR) 3:16, 1989), encephalopathy (Ross et al., Neurotoxicology 9:581, 1988), ankylosis

(Mahowald et al., Arthritis Rheum. 31:1390, 1988), autoimmunity (Roberts et al., J. Comp. Pathol. (England) 100:391, 1989), systemic lupus (Mendlovic et al., Proc. Natl. Acad. Sci. USA 85:2260, 1988), epilepsy (King et al., Epilepsia 30:257, 1989), insulin-dependent diabetes (Gottlieb et al., Diabetes Care 11:29, 1988), connective tissue disease (Rosenberg N.L., Arthritis Rheum. 31:806, 1988), and copper metabolism (Shiraishi et al., Biol. Neonate (Switzerland) 54:173, 1988). Rats have also served as successfully animal models for studying cystic fibrosis (Hunsinger et al., Med. Hypotheses (England) 28:81, 1989), osteoporosis (Yamazaki et al., J. Bone Miner Res. 4:13, 1989), and obesity and hypertension (Crandall et al., J. Appl. Physiol. 64:1094, 1988).

Animal species other than rodents have also been used successfully to study human diseases. Pigs have been used to investigate *E. coli* infection (Sarmiento et al., Am. J. Vet. Res. 49:1154, 1988), acetaminophen (Henne-Bruns et al., Res. Exp. Med. (Germany) 188:463, 1988), asthma (Karol M.H., Bull. Eur. Physiopathol. Respir. (England) 23:571, 1987), and herpes virus infection (Hsiung et al., Antiviral Res. (Netherlands) 12:239, 1989), while sheep have been employed to study periodontitis (Ismaiel et al., J. Periodontol. 60:279, 1989) and chronic venous disease (Jessup et al., J. Vasc. Surg. 8:569, 1988). Chronic subluxation (De Boer et al., J. Manipulative Physiol. Ther. 11:366, 1988) and respiratory distress syndrome (Lachmann B., Dev. Pharmacol. Ther. (Switzerland) 13:164, 1989) have been studied in adult and fetal rabbits, while hyperoxaluria (Danpure et al., J. Inherit. Metab. Dis. (Netherlands) 12:403, 1989) has been studied in felines. Arthritis has been studied in both rats (Barthold et al., Ann. NY Acad. Sci. 539:264, 1988) and rhesus monkeys (Pritzker et al., PR Health Sci. J. (Puerto Rico) 8:99, 1989). HIV infection has been intensively studied using such animal models as chimpanzees, gibbons, mice, rabbits, baboons, and rhesus monkeys (Spertzel R.O., Antiviral Res. (Netherlands) 12:223, 1987).

Where suitable animal models are not available, however, the study of the disease, and its treatment, may be severely hampered. In particular, the study of human hematopoiesis has been hampered by the lack of an animal model for studying hematopoietic cells. The lack of a suitable animal model is especially keen today with the emergence of gene therapy for human diseases. Presently, the only available means of evaluating a given gene therapy is a clinical trial - a costly, complex and time-consuming endeavor. Accordingly, an animal model for studying the introduction of genetic material into the human pluripotent stem cell would be a major advance for the field.

Hematopoiesis, the formation of mature blood cells, involves a complex scheme of multilineage differentiation (Metcalf, Nature 339:27-30, 1989). In the bone marrow, pluripotent stem cells proliferate and differentiate into progenitor cells which then develop into different types of mature blood cells (Gordon et al., Bone Marrow Transplant 4:335, 1989; Dexter et al., Ann. Rev. Cell Bio. 3:423, 1987). Indeed, the pluripotent stem cell, identified by the presence of the CD34 hematopoietic cell surface antigen, is capable of differentiating and giving rise to cells of all hematopoietic lineages (Sprangruide et al., Science 241:58, 1988).

Two animal models, based on the severe combined immunodeficiency (SCID) mouse, have been developed to study the growth and differentiation of human bone marrow cells, the source of hematopoietic cells. Both of these models, however, have drawbacks and disadvantages which limit their usefulness. In the first system, established by John Dick, human cytokines are supplied by systemic administration to the SCID mice transplanted with human bone marrow (Lapidot et al., Science 255:1137-1141 (1992)). (See also, Dick et al., Sem. Immunol. 3:367-378 (1991).) This system is very expensive, requiring 3-times a week injections of purified recombinant cytokines.

The second system, developed by Jim McCune, involves the surgical implantation of human fetal tissues into SCID mice

(McCune et al., Science 241:1632-1639 (1988); and Kaneshima et al., Nature 348:561-562 (1990)). In the McCune system, human fetal liver, human fetal thymus and/or human fetal lymph nodes are implanted into SCID mice. These mice support the differentiation of mature human T cells and B cells. The McCune system has also succeeded in sustaining active human hematopoiesis in SCID mice implanted with human fetal bone (Kyoizumi et al., Blood 79:1704-1711 (1992)). The surgical procedures, however, which must be conducted on each mouse with the McCune system are time consuming and costly. In addition, the availability of fetal tissue is limited and its use makes this approach controversial.

SUMMARY OF THE INVENTION

Accordingly, it is one object of the present invention to provide an animal model that can be used as a pre-clinical assay to evaluate different proposed gene therapies by testing whether the therapy effectively corrects the given deficiency.

It is a further object of the present invention to provide an inexpensive, straight forward, and versatile means of studying the growth and differentiation of human bone marrow cells.

It is another object of the present invention to provide a human microenvironment in mice using an immortalized human cell line.

Various other objects and advantages of the present invention will be apparent from the drawings and the following description of the invention.

To accomplish these objectives, the present invention relates to a method of engrafting human or non-human primate stem cells in a mouse to create an animal model useful for the study of hematopoietic cells. In one embodiment, the cells are primitive stem cells that have the capability of differentiating into all hematopoietic cells. In a preferred embodiment, the stem cells are CD34+. In a particular embodiment, the method comprises irradiating the mouse with a

lethal or non-lethal dose of radiation; administering to the irradiated mouse immortalized human or non-human primate bone marrow stromal cells which express cytokines supporting growth of stem cells; and administering to the irradiated mouse purified human or non-human primate stem cells.

In another embodiment, the present invention relates to a method of testing the effectiveness of a gene therapy protocol against an infectious agent such as HIV. The method comprises irradiating a first and a second mouse with a lethal or non-lethal dose of radiation; administering to the irradiated mice immortalized human or non-human primate bone marrow stromal cells which express cytokines supporting growth of CD34+ stem cells; introducing a proposed therapeutic gene segment into a first group of human CD34+ stem cells; purifying the cells from the first group of CD34+ stem cells expressing the gene segment; introducing a control gene segment into a second group of human CD34+ stem cells; administering the purified CD34+ stem cells expressing the gene segment to the first irradiated mouse and administering the control cells to the second irradiated mouse; challenging both irradiated mice with the retrovirus; and comparing the survival of the human CD34+ stem cells from the two mice.

In a further embodiment, the present invention relates to an animal model for the study of human hematopoiesis. This model comprises a rodent into which human or non-human primate CD34+ stem cells have been engrafted in the present of immortalized human or non-human primate bone marrow stromal cells. In a preferred embodiment, the rodent is a mouse, such as a SCID mouse.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the relative expression of cytokine mRNA transcripts in the human stromal cell line Lof(11-10).

Figure 2 shows the survival of lethally irradiated SCID mice reconstituted with the human stromal cell line Lof(11-10).

Figure 3 shows the detection of Lof(11-10) cells in SCID mice by DNA polymerase chain reaction (PCR).

Figure 4 shows the detection of human and monkey CD34 derived cells in the bone marrow compartment of SCID mice by reverse transcriptase (RT)-dependent RNA PCR. Lanes 1-4 show mice receiving 1 x 10^7 Lof(11-10) cells by intraperitoneal injection (i.p.) plus 3 x 10^5 human CD34+ cells by intravenous injection (i.v.) Lane 5 shows mice receiving 1 x 10^7 Lof(11-10) cells i.v. plus 2 x 10^5 monkey CD34+ cells i.v. Lanes 6-8 show mice receiving 1 x 10^7 Lof(11-10) cells i.p. plus 2 x 10^5 monkey CD34+ cells i.v.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method of engrafting human or non-human primate pluripotent stem cells in a rodent. Using severe combined immunodeficiency (SCID) mice, the inventors developed a method for engrafting human or non-human primate pluripotent CD34+ stem cells into the SCID mouse. Although mice were utilized in the development of the present method, any rodent may be used. Preferred rodents include SCID mice; these mice have mutated *scid* genes which prevent production of mature T cells and B cells by the mice. All other hematopoietic lineages are produced as normal in these mice.

Pluripotent stem cells can be defined functionally as well as phenotypically. Functionally, pluripotent stem cells are those hematopoietic cells having the capability of multilineage differentiation and self-renewal. Pluripotent stem cells can also be defined phenotypically by cell surface markers. For example, human stem cells have been characterized as CD34+ CD38-, HLA-DR-, THY+, and Rh123-.

In the present method, human and non-human primate pluripotent stem cells are engrafted in a rodent, preferably a mouse. To engraft human and non-human primate stem cells so that the cells are maintained and differentiated in the mouse, the mouse is first irradiated with a lethal or non-lethal dose of radiation. For example, each mouse can be

exposed to 200 or 400 rads of gamma irradiation. Once irradiated, the mice are given a dose of immortalized cells which support the growth of stem cells. In one embodiment, the supportive cells are immortalized bone marrow stem cells which express cytokines. Finally, the mice are given an injection of purified human or non-human primate CD34+ cells.

The cells engrafted into the rodent are preferably CD34+ stem cells obtainable from bone marrow, peripheral blood or cord blood. These cells can be isolated from human or non-human primate bone marrow or blood using methods known in the art. Other cells useful in this engraftment method include, but are not limited to, fetal liver and fetal thymus.

Immortalized bone marrow stromal cells are administered to the irradiated mice to provide, within the mice, a substratum on which later administered primitive hematopoietic cells, such as CD34+ stem cells, can adhere. These stromal cells also nurture such cells by producing the cytokines necessary for their growth and differentiation. The immortalized bone marrow stromal cells serve as a microenvironment within the mouse where stem cells engraftment, differentiation, and self-renewal can occur.

The immortalized stromal cells preferably produce human cytokines. Suitable immortalized bone marrow stromal cells include, but are not limited to, the Lof(11-10) cell line. The Lof(11-10) cell line is preferred because these cells naturally produce human cytokines and, therefore, can support the growth of CD34+ cells *in vitro*. The Lof(11-10) cell line produces Il-1α, IL-1β, IL-6, IL-8, GM-CSF, M-CSF, G-CSF, and SCF (stem cell factor). In addition, these cells are immortalized using the SV40 large T-cell antigen and, unlike transformed cells, do not produce tumors when administered to mice.

Like the Lof(11-10) cell line, the immortalized bone marrow stem cells employed in the present invention may naturally produce the desired cytokines. Alternatively, the bone marrow stem cells can be genetically engineered, using standard methods known in the art, to produce the desired

cytokines or other desired supporting components. For example, the Lof(11-10) cell could be modified to further produce such human cytokines as IL-3, additional SCF, IL-7 or IL-2.

The immortalized bone marrow stromal cells and the purified CD34+ cells are administered in amounts sufficient to promote engraftment of the CD34+ cells. Preferably, 0.1-5.0 x 10^7 bone marrow stromal cells are administered to each rodent, more preferably each rodent is given 0.1, 0.5, 1.0 or 5.0 x 10^7 cells. Preferably, 0.1 - 5.0 x 10^5 CD34+ cells are given to each rodent. More preferably, each rodent is given a dosage of 0.1, 0.5, 1.0 or 5.0 x 10^5 CD34+ cells.

These two populations of cells can be administered to the mouse simultaneously or sequentially. Preferably, the immortalized bone marrow stromal cells are administered first intraperitoneally (i.p.), followed by sequential administration of the purified CD34+ cells intravenously (i.v.). For example, the purified CD34+ cells can be administered about 10 days after the immortalized bone marrow stromal cells are administered, preferably 2-7 days after the immortalized bone marrow stromal cells are administered.

The present invention is an inexpensive, straight forward, and versatile system for engrafting human and non-human primate CD34+ cells in the mouse animal model. The method is relatively inexpensive as the immortalized bone marrow stromal cells are capable of growth in cell cultures. For example, Lof(11-10) cells can be grown to 1 x 10^7 per T225 flask, which is enough cells to introduce into one mouse. The method is straight forward in that the injection of the immortalized bone marrow stromal cells is easy to perform and provides a continuous supply of human cytokines in the mouse. Finally, the method is versatile. The immortalized bone marrow stromal cells can be made to express additional human growth factor, surface receptors and/or cytokine genes.

Mice engrafted with human stem cells such as CD34+ cells, according to the present invention, produce all lineages of

human blood cells. The engrafted CD34+ cells are able to proliferate and differentiate into all blood cell lineages including human T cells and human B cells, thereby establishing a human hematopoietic microenvironment in the mouse. This animal model for human hematopoiesis has several uses. For example, the mice of the present invention can be used as a model for studying infectious diseases *in vivo*. This model can also be used to screen compounds for antiviral activity *in vivo*. This mouse model can also be used to assay for hematopoietic growth factors, immune modulators and/or immune toxins. Finally, mice engrafted with human CD34+ cells can be used in the production of human monoclonal antibodies and human cytolytic T cell clones.

The present invention also relates to a method of testing the effectiveness of gene therapy protocols against infectious agents such as human immunodeficient virus (HIV), human cytomegalovirus (CMV), human papilloma virus (HPV), and Epstein-Barr virus (EBV). In this testing method, putative therapeutic genetic material is introduced into the human CD34+ cell and the CD34+ cells are then engrafted in a rodent to determine whether the introduction of the genetic material prolongs the survival of the CD34+ cells and their progeny *in vivo*. The genetically engineered CD34+ cells are engrafted in, for example, a mouse as described above. The mouse is then challenged with the infectious agent and survival of the human CD34+ cells and their progeny is determined.

For example, the present testing method can be used to determine the effectiveness of a gene therapy protocol against HIV, the causative agent of AIDS. An effective gene therapy protocol against HIV infection would confer resistance against HIV to cells *in vivo*. If resistance to HIV infection can be conferred upon cells *in vivo* using a particular gene therapy protocol, further studies would be warranted to optimize the therapeutic benefits of the molecular intervention in AIDS patients.

In the method of the invention, a proposed anti-HIV gene is introduced into human CD34+ cells and the cells expressing

the gene are then purified. Introduction of the proposed anti-HIV gene into CD34+ cells can be accomplished by techniques standard in the art such as a viral-mediated vector system. Purification of the expressing CD34+ cells can be easily done by techniques in the art such as either drug selection or surface expression of genes co-introduced with the anti-HIV gene. A control gene is introduced into a separate aliquot of human CD34+ cells. The control gene, for example, contains the proposed anti-HIV gene but has a frameshift which abrogates expression of the proposed anti-HIV gene.

Effects of the proposed anti-HIV gene and the control anti-HIV gene can be analyzed using either one or two populations of mice that have been irradiated with a lethal or non-lethal dose of radiation and given a dose of immortalized human bone marrow stromal cells which express cytokines supporting the growth of CD34+ cells. If two mice populations are used, one population is administered the human CD34+ cells expressing the proposed anti-HIV gene and the other is given the human CD34+ control cells. The mice are then challenged with HIV. The half-life and survival of the human CD34+ cells and their progeny which express the proposed anti-HIV gene are compared with the CD34+ control cells. These two populations can be distinguished using techniques familiar in the art such as a limited dilution PCR of cells derived from the spleen and bone marrow with primers specific for each vector. In this way, the number of cells which persist can be determined as a function of time, and the half-life of cells transduced with each vector can be assessed.

The present system can also be used to assay for the successful introduction of a therapeutic gene into the true stem cells, which are capable of self-renewal and life-long expression. This can be determined by PCR analyses of human spleen colonies growing in SCIDs 14 days after CD34+ cells injection. These colonies represent the earliest assayable cells derived from the pluripotent stem cell. Introduction

of an effective therapeutic into this cell population would ensure a one-time gene therapy in recipient patients.

This is important because only 0.1 to 1 percent of the CD34+ cells are true pluripotent stem cells; the remaining 99 percent of the CD34+ cells are committed progenitors which will give raise to various blood lineages. Thus, the expression of a therapeutic gene in the CD34+ population does not necessarily mean that the therapeutic is in the stem cell. Although the expression of a therapeutic would be useful in the mature cells in the blood, the life-span of these mature cells is finite. Introduction of the therapeutic into the pluripotent self-renewing stem cell insures a continuous supply of committed progenitors which in turn insures a continuous supply of mature cells in the blood armed with the therapeutic.

By way of example, in the case of HIV, the anti-HIV gene would be present in the mature T cells and monocytic cells of an HIV infected patient, potentially allowing that individual to lead a normal life in spite of harboring the AIDS virus. At the present time, a functional assay for the introduction of a therapeutic into the stem cell is nonexistent and would have to wait for the outcome of a clinical trial or a bone marrow transplant in primates. The use of the spleen colony assay of the present invention for human cells within the SCID spleen would greatly facilitate the development of the procedures to assay for the introduction of the therapeutic gene into human stem cells, insuring the continuous supply of committed progenitors and mature cells armed with the antiviral gene.

The present invention further relates to a method of treating patients exposed to radiation to reduce recovery time. Radiation is currently used to treat different forms of cancer including leukemia and to treat patients undergoing bone marrow transplantations. When patients are exposed to radiation for bone marrow transplants, all of their bone marrow is destroyed, including their progenitor cells (which give rise to the mature cells in the blood) and the stromal

cells (which nurture the stem and progenitor cells to renew, grow and differentiate). The window of time (1 to 2 months) between whole body irradiation and the growth/differentiation of the "newly" introduced bone marrow cells into the patient is the most critical time in bone marrow transplantation. This is the time when the patient is most susceptible to either rejection (host versus graft disease) or opportunistic infections (due to an incompetent immune system).

Cytokine therapy has shown promise when used during the period of time between whole body irradiation and growth/ differentiation of the new cells, suggesting that the establishment of a functional stromal layer is essential to supply the cytokines needed to allow stem and progenitor cell growth. The "newly" introduced bone marrow cells will, in time, establish their own stromal layer; however, the introduction of a stromal cell line expanded *in vitro* and introduced with the "new" marrow may reduce this window of uncertainty. The stromal cells can be obtained from the patients' own marrow or from their donor by procedures similar to those used to establish the Lof(11-10) cell line.

For the purposes of illustrating a preferred embodiment of the present invention, in the following non-limiting examples, the immortalized human stromal cell line Lof(11-10) was used to reconstitute SCID mice with human and monkey CD34+ cells. It is, however, to be understood that the discussion generally applies to the reconstitution of any mouse with human cells capable of producing cytokines.

EXAMPLES

Example 1: Lof(11-10) Cells Produce Cytokines Which Support *In Vitro* CD34+ Cell Growth

Lof(11-10) is a human immortalized stromal cell line established from human bone marrow by transfection of a plasmid containing a SV40 large T antigen (pSV3gt) as described by S.W. Gartner (Establishment and Characterization of Human Bone Marrow Stroma-Derived Cell Lines Transformed Following Transfection with Cloned SV40 Early Region DNA (1984) (Ph.D. dissertation, Stanford University). To determine whether Lof(11-10) cells produce cytokines which are required to support cell growth, Lof(11-10) cells were grown in T162 tissue culture flasks for 10 days. The expression of cytokine mRNA transcripts in the Lof(11-10) cultures was then detected using reverse transcriptase (RT)-dependent RNA PCR analysis.

Briefly, total RNA was isolated from the cultured Lof(11-10) cells using the RNAzol B RNA isolation solvent (Tel-Test) according to manufacturer's instructions. Ten micrograms of purified RNA were incubated at 37° C for 30 minutes with 100 units of RNase-free DNase in 0.1 M sodium acetate, 5 mM $MgCl_2$ in a 50 μl reaction, followed by phenol/chloroform extraction and ethanol precipitation. Samples of 5 μg of DNase-treated RNA were converted to cDNA by primer extension with 0.1 mg/ml random primers, and 600 units of Moloney murine leukemia virus RT (Bethesda Research Laboratories). The RNA and the random primers were heated to 70° C for 10 minutes followed by quick cooling on ice. The mixture was then incubated for 1 hour at 37° C in a 40 μl reaction volume containing 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 6 mM $MgCl_2$, 10 mM DTT and 750 μM dNTPs. The RT reaction was terminated by heating at 90° C for 5 minutes before PCR amplification.

Aliquots of the DNase-treated RNA, with and without conversion to cDNA by reverse transcriptase, were amplified with 0.5 μM sequence specific primer pairs in a 50 μl

reaction volume containing 2 μl of reverse-transcribed cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 mM dNTPs, and 1.5 units of Taq DNA polymerase (Perkin-Elmer/Cetus) for 28 cycles. The specific primer pairs used were as follows:

IL-1α: 5'-ATGGCCAAAGTTCCAGACATGTTTG (SEQ ID NO:1)
3'-GGTTTTCCAGTATCTGAAAGTCAGT (SEQ ID NO:2)

IL-1β: 5'-ATGGCAGAAGTACCTAAGCTCGC (SEQ ID NO:3)
3'-ACACAAATTGCATGGTGAAGTCAGTT (SEQ ID NO:4)

IL-6: 5'-ATGAACTCCTTCTCCACAAGCGC (SEQ ID NO:5)
3'-GAAGAGCCCTCAGGCTGGACTG (SEQ ID NO:6)

IL-8: 5'-ATGACTTCCAAGCTGGCCGTGCT (SEQ ID NO:7)
3'-TCTCAGCCCTCTTCAAAAACTTCTC (SEQ ID NO:8)

GM-CSF: 5'-ATGTGGCTGCAGAGCCTGCTGC (SEQ ID NO:9)
3'-CTGGCTCCCAGCAGTCAAAGGG (SEQ ID NO:10)

G-CSF: 5'-GAGTGTGCCACCTACAAGCTGTGCC (SEQ ID NO:11)
3'-CCTGGGTGGGCTGCAGGGCAGGGGC (SEQ ID NO:12)

M-CSF: 5'-GAGGAGGTGTCGGAGTACTGTAGCCACATG (SEQ ID NO:13)
3'-CATAGAAAGTTCGGACGCAGGCCTTGTCATG (SEQ ID NO:14)

TGFα: 5'-ATGGTCCCCTCGGCTGGACAG (SEQ ID NO:15)
3'-GGCCTGCTTCTTCTGGCTGGCA (SEQ ID NO:16)

SCF: 5'-CCACTGTTTGTGCTGGATCGCAGCGCTGCC (SEQ ID NO:17)
3'-GTCATCCACTATATTCACAAGTTTGTCTATG (SEQ ID NO:18).

The PCR profile consisted of 94° C for 1 minute, 60° C for 1 minute, and 72° C for 3 minutes, for the denaturation, annealing, and extension reactions, respectively. The samples were incubated at 94° C for 3 minutes before the start of the PCR profile and a final extension time of 20 minutes at 72° C was provided after the 28th PCR cycle.

The amplified products were separated on 1.2% agarose gels in the presence of 0.5 μg/ml ethidium bromide, transferred to nylon membrane, and probed with ^{32}P end-

labeled oligodeoxyribonucleotides having the following sequences:

IL-1α: CCTTCTATCATGTAAGCTATGGCCCACTCC (SEQ ID NO:19)
IL-1β: GGTCCATATGAACTGAAAGCTCTCCACCTC (SEQ ID NO:20)
IL-6: GAAAAAGATGGATGCTTCCAATCTGG (SEQ ID NO:21)
IL-8: GAACTGAGAGTGATTGAGAGTGGACCACAC (SEQ ID NO:22)
GM-CSF: CGCCTGGAGCTGTACAAGCAGGGCCTGCGG (SEQ ID NO:23)
G-CSF: CTGCAGGCCCTGGAAGGGATCTCCCCCGAG (SEQ ID NO:24)
M-CSF: GAGGACACCATGCGCTTCAGAGATAACACC (SEQ ID NO:25)
TGFα: GGAACCTGCAGGTTTTTGGTGCAGGAGGAC (SEQ ID NO:26)
SCF: CCAAAAGACTACATGATAACCCTCAAATATG (SEQ ID NO:27).

The probe sequences represented regions internal to, and not overlapping with, primers used for amplification. Hybridization signals were quantified directly from the nylon membrane using a PhosphorImager scanner with ImageQuant software form Molecular Dynamics (Sunnyvale, CA).

The results, shown in Figure 1, demonstrate that Lof(11-10) cells produce cytokines, such as IL-1B, IL-8, M-CSF and B-ACTIN, which support the growth of cells, such as the CD34+ stem cell. Additional cytokines with short-lived mRNAs like Il-6, GM-CSF, and G-CSF are also expressed in the present of protein synthesis inhibitors.

Cytokine expression by Lof(11-10) cells, 5637 cells (a human bladder primary carcinoma) and GCT cells (a human fibrous histicytoma) were quantitated using ELISA immunoassays. The 5637 and GCT cell lines are available from American Type Culture Collection (ATCC), Rockville, MD. Briefly, measurement of cytokines was performed with culture supernatant using ELISA kits from R & D Systems (Minneapolis, MN) according to the manufacturer's specifications. The results of the ELISA are shown below in Table 1.

TABLE 1

	Cytokines (pg/ml)			
Condition Media	IL-α	IL-6	GM-CSF	SCF
GCT	115	>300	>500	<10
5637	>250	>300	>500	250
Lof(11-10)	200	3665	150	70

Example 2: Lof(11-10) Cells Support *In Vitro* CD34+ Progenitor Cells

Initially, attempts were made to establish a stroma-dependent long-term bone marrow culture using the Lof(11-10) cells as a stromal feeder layer. However, the rapid growth of the Lof(11-10) cells depleted the nutrients from these cultures, and thus inhibited the proliferation and differentiation of the CD34+ hematopoietic progenitors in the co-culture system. To circumvent this problem, a 14-day hematopoietic methylcellulose culture containing 20% conditioned media from the parent Lof(11-10) culture was used to support CD34+ cell growth and differentiation into cell populations designated as CFU-M, CFU-G, and CFU-GM colonies. Colony growth was detected using standard *in vitro* assays. The Lof(11-10) cells supported colony formation similar to the conditioned media obtained from either the 5637 or the GCT cell lines (Tables 2 and 3). Both the 5637 and GCT lines cannot be used in the SCID mouse because of their transformed phenotype, which causes tumor formation. The Lof(11-10) cells are immortalized and will not form tumors in mice.

Table 2: Human CD34+ Bone Marrow Derived Cells

Conditioned Media	BFU-E	CFU-GM
GCT	72+/-12	43+/-5
5637	77+/-6	49+/-2
Lof(11-10)	55+/-5	43+/-1

Table 3: Monkey CD34+ Bone Marrow Derived Cells

Conditioned Media	CFU-GM
GCT	28+/-5
5637	23+/-4
Lof(11-10)	22+/-2

Example 3: Reconstitution of SCID Mice with Lof(11-10) Cells

The objective in reconstituting SCID mice was to establish a microenvironment that would provide a substratum on which primitive human CD34+ hematopoietic cells could adhere and that would nurture the human CD34+ cells by producing human cytokines. To reconstitute the SCID mice, each mouse was gamma irradiated with either 200 rads (non-lethal) or 400 rads (lethal). The mice were irradiated to provide an internal space within which the CD34+ cells could repopulate. Four hours after irradiation, the mice were injected intraperitoneally (i.p.) with 5×10^7 Lof(11-10) cells obtained as described above. As controls, two SCID mice were injected with Iscove's media without Lof(11-10) cells.

Five to seven days after the injection of Lof(11-10) cells, each mouse was given an intravenous (i.v.) injection of either 3 to 5 purified human CD34+ cells or 2 to 5 purified monkey CD34+ cells. The bone marrow-derived CD34+ cells were obtained from the posterior iliac crest under

local anesthesia from healthy donors with informed consent. Briefly, bone marrow aspiration was performed into a 20 ml syringe containing EDTA as an anticoagulant. Bone marrow cells were diluted 1:2 with PBS and were subjected to ficoll-hypaque density gradient centrifugation. Interphase mononuclear cells were washed twice in PBS and were further purified using magnetic beads linked with the high affinity CD34 monoclonal antibody K6.1. The monoclonal antibody K6.1 was produced by fusing SP-2/0-AG14 plasmacytoma cells ((ATCC), Rockville, MD) with splenocytes from a BALB/cByJ mouse (Jackson Laboratory, Bar Harbor, ME) which had been immunized with viable KG-1a cells (ATCC, Rockville, MD). After magnetic selection, beads were chemically dissociated from the cells and the cells were referred to as CD34-positive cells (Kirshenbaum et al., J. Immunol. 14:1410, 1991).

The time interval between the injection of the two cell populations was to allow engraftment of the Lof(11-10) cells before the introduction of the CD34+ cells. By first reconstituting the SCID mice with the Lof(11-10) cells, human cytokines needed to nourish the CD34+ hematopoietic pluripotent stem, progenitor and blood lineage precursor cells were supplied. In addition, the Lof(11-10) cells served as a substratum for cell "homing" and the early attachment of the CD34+ cells (1 to 10 days) during reconstitution.

In the initial experiments, SCID mice were irradiated with 400 rads. Mice (n=2) injected with media without Lof(11-10) cells died at 7 days, whereas mice (n=5) receiving Lof(11-10) cells lived to 50 days post-irradiation. (See Figure 2). This suggests that the Lof(11-10) cells reconstituted the mice and transiently protected the mice from the lethal irradiation resulting in a 6-fold increase in survival of the mice.

The presence of Lof(11-10) cells in the SCID mice was confirmed using DNA PCR. Briefly, DNA was purified from both the spleen and bone marrow cells from either control or

Lof(11-10)-injected mice. Aliquots (1 μg) were amplified with 0.5 μM sequence-specific primer pairs for either T-antigen or mouse ribosomal 28S gene, in a 50 μl reaction volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 mM dNTPs, and 1.5 units of Taq DNA polymerase for 28 cycles. The specific primer pairs used were as follows:

SV40 large T: 5'-CCTGGTGTTGATGCAATGTACTGCAAAC (SEQ ID NO:28)

3'-CACAAACAATTAGAATCAGTAGTTTAACAC (SEQ ID NO:29)

MOUSE 28s rRNA: 5'-GTCGACCAGTTGTTCCTTTGAGGTCCG (SEQ ID NO:30)

3'-GCTGGACAAGCAAAACAGCCTTAAATCG (SEQ ID NO:31).

The PCR profile consists of 94° C for 1 minute, 60° C for 1 minute, and 72° C for 3 minutes, for the denaturation, annealing, and extension reactions, respectively.

The amplified products were separated on 1.2% agarose gels in the presence of 0.5 μg/ml ethidium bromide, transferred to nylon membranes, and probed with ^{32}P end-labeled oligonucleotides for either T-antigen or mouse 28S gene, respectively. The probes used were as follows:

SV40 large T: CGATTGCTTTAGAATGTGGTTTGGACTTGATC (SEQ ID NO:32)

MOUSE 28s rRNA: CCTCCCTGTCTCTTTTATGCTTGTGATC (SEQ ID NO:33).

The probe sequences represented regions internal to, and not overlapping with, primers used for amplification. Hybridization signals were quantified directly from the nylon membranes using a PhosphorImager scanner with ImageQuant software form Molecular Dynamics (Sunnyvale, CA). The results of the DNA PCR experiments are shown in Figure 3.

The presence of circulating human IL-6 demonstrates that the Lof(11-10) cells are functional and expressing their repertoire of human cytokines. The measurement of human IL-6

was done on plasma obtained from the blood of animals, by ELISA analysis. (Table 4.)

Table 4: Quantitation of Human IL-6 from Reconstituted SCID Plasma by ELISA

Expt #	Condition[1]	IL6 (pg/ml)			
1	Control[2] (200 rad, 4-6 wks)	ND[3]			
	Lof(11-10) reconstituted[2] (200 rad, 4-6 wks)	39			
		Mouse # 1	2	3	4
2	Monkey CD34 reconstituted (200 rad, 6 wks)	ND	ND	ND	ND
	Lof(11-10)+ monkey CD34 reconstituted[2] (200 rad, 4-6 wks)	25	11	ND	16

[1] 50 x 10^6 Lof(11-10) injected i.p.; 0.5 10^6 monkey CD34+ injected i.p.

[2] 25-75 ml of plasma from 5 mice were pooled for analysis

[3] ND = non detected, OD<negative standard control value

Additional evidence that the human cytokines expressed from the injected Lof(11-10) cells are circulating and potentially capable of supporting the engraftment of intravenously injected human CD34+ cells comes from their influence on the bone marrow progenitor cells of the mice, as measured by methylcellulose colony formation (Table 5). Whereas irradiation reduces the number of colonies in methylcellulose assays, the animals inoculated with Lof(11-10) cells showed a restoration of colony formation in spite of irradiation, suggesting that the Lof(11-10) cells are expressing radioprotective cytokines that can reverse the negative effects of irradiation on mouse progenitor cells P20.

TABLE 5: COLONY FORMATION FROM SCID MOUSE BONE MARROW

Expt. #	Treatment	Time After Radiation	CFU-GM Colonies			Relative Percentage
			SCID #1	SCID #2	SCID #3	
1	No radiation	6 Wks	74+/-15	55+/-7	--	100
	200 rads		21+/-7	10+/-7	28+/-8	30
	200 rads + Lof(11-10)		53+/-9	34+/-11	93+/-12	93
2	No radiation	6 Wks	57+/-33	--	--	100
	200 rads		10+/-6	--	--	17
	200 rads + Monkey CD34		64+/-25	57+/-21	122+/-18	142
3	No radiation	8 Wks	152+/-21	--	--	100
	200 rads		15+/-3	--	--	10
	200 rads + Monkey CD34		84+/-18	79+/-9	--	54
	200 rads + Lof(11-10) + Monkey CD34+		67+/-25	169+/-7	112+/-15	76

The ultimate demonstration that the Lof(11-10) cells will support human and primate CD34+ cells comes from the fact that secondary intravenous injection of the animals results in the presence of CD34+ cells within the mouse bone marrow after 3 weeks. (Figure 4.)

The spleen and bone marrow compartments of the SCID mice were analyzed to determine whether Lof(11-10) or CD34+ engraftment occurred in these compartments. The Lof(11-10) cells when injected intraperitoneally into the irradiated mice were found to migrate to the spleens. (Figure 3.)

The CD34+ cells detected in the bone marrow of the mice were derived from the human CD34+ cells as indicated by the fact that they are class 2 positive, expressing HLA-DR alpha transcripts by reverse transcriptase dependent RNA PCR. Lof(11-10) cells are class 1 positive, but class 2 negative. Additional evidence that the PCR signals observed in the bone marrow are CD34+ cells derived comes from the lack of detection of SV40 T-antigen by DNA-PCR in these preparations. Further characterization of state of differentiation of the

CD34+ cells within the bone marrow will be done by following the expression of various cell surface receptors using reverse transcriptase dependent RNA PCR.

* * * * *

All publications mentioned hereinabove are hereby incorporated in their entirety by reference.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Mosca, Joseph D.
Gartner, Suzanne
Kessler, Steven
Hall, Eric
Kaushal, Sumesh

(ii) TITLE OF INVENTION: System For The Maintenance, Growth And Differentiation Of Human And Non-Human Primate Pluripotent Stem, Progenitor And Mature Bone Marrow Cells

(iii) NUMBER OF SEQUENCES: 33

(iv) CORRESPONDENCE ADDRESS:
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(C) CITY: Washington
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(E) COUNTRY: USA
(F) ZIP: 20005

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/352,957
(B) FILING DATE: 09-DEC-1994
(C) CLASSIFICATION:

(vii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Fordis, Jean B.
(B) REGISTRATION NUMBER: 32,984
(C) REFERENCE/DOCKET NUMBER: 04995.0010-00000

(viii) TELECOMMUNICATION INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGGCCAAAG TTCCAGACAT GTTTG 25

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGACTGAAAG TCTATGACTT CTTGG 25

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATGGCAGAAG TACCTAAGCT CGC 23

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TTGACTGAAG TGGTACGTTA AACACA 26

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATGAACTCCT TCTCCACAAG CGC 23

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GTCAGGTCGG ACTCCCGAGA AG 22

Detection in the Bone Marrow Compartment by RT-Dependent RNA PCR

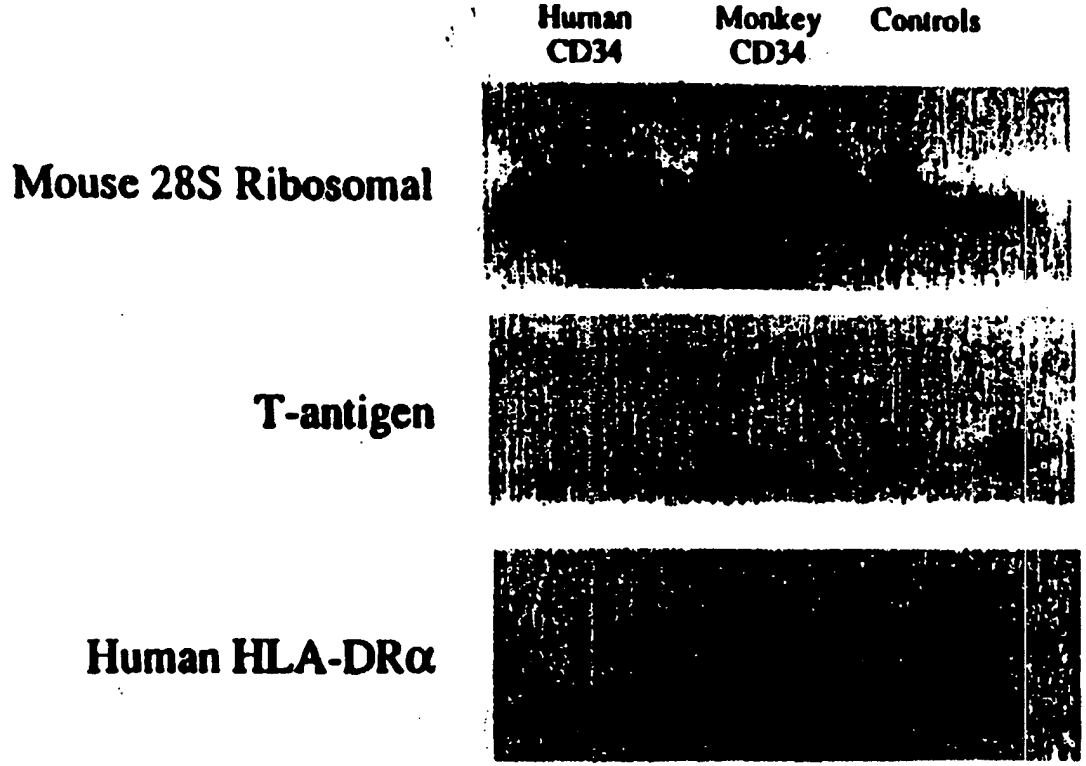
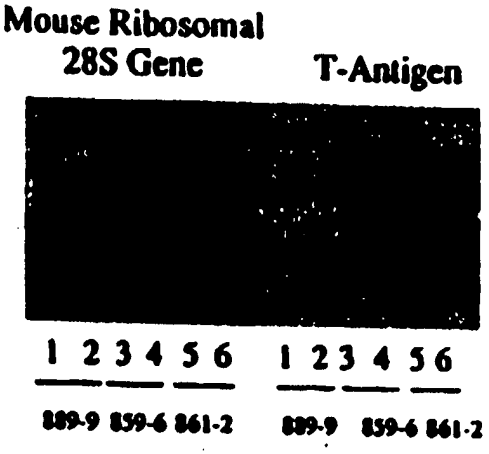


FIGURE 4

Detection of Lof (11-10) Cells in SCID Mice by DNA PCR



Mouse #889-9 = Control (200 rads, 28 days)

Mouse #859-6 + 861-2 = Lof (11-10) reconstituted (200 rads, 28 days)

Lanes 1, 3 & 5 = Mouse bone marrow samples

Lanes 2, 4 & 6 = Mouse spleen samples

FIGURE 3

Survival of Lethally Irradiated SCID Mice Reconstituted with the Human Stromal Cell Line Lof(11-10)

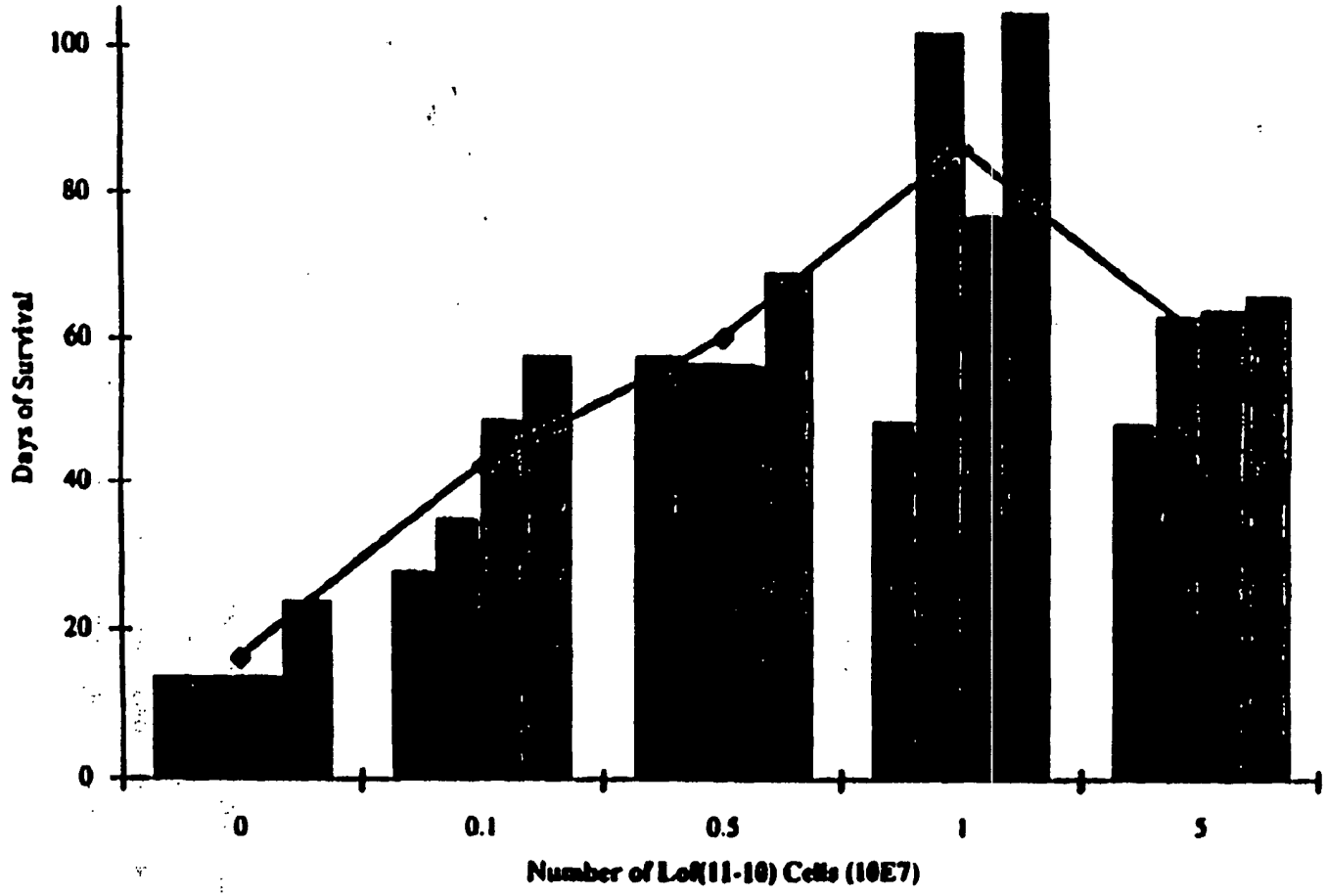


FIGURE 2

Mice were irradiated with 400 rads. Four mice in each group were injected i.p. with the indicated number of cells.

Relative Expression of Cytokine mRNA Transcripts in Human Stromal Cell Line Lof(11-10)

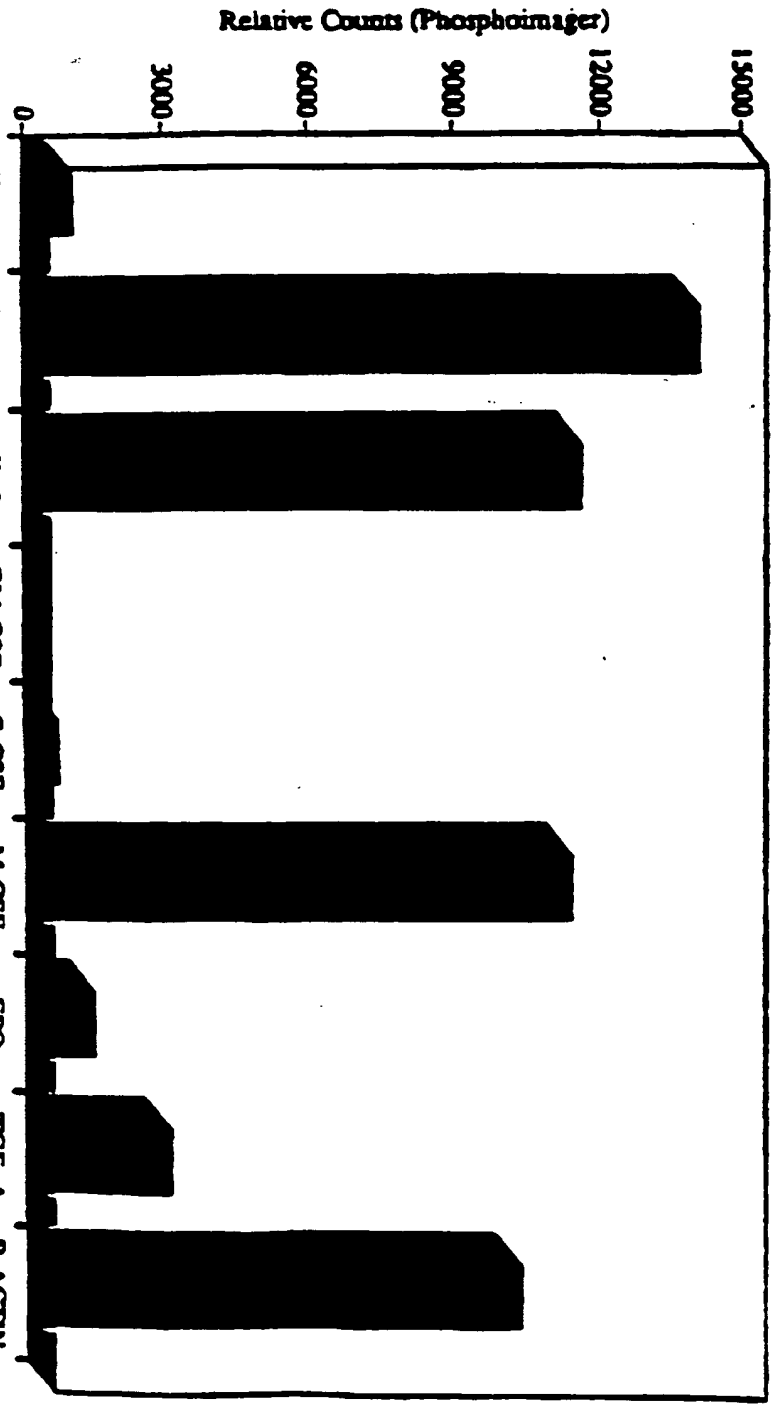


FIGURE 1

19. The animal model according to claim 18, wherein the mouse is a SCID mouse.

20. The animal model according to claim 16, wherein the immortalized bone marrow stromal cells are transfected with SV40 large T-cell antigen.

21. The animal model according to claim 20, wherein the immortalized bone marrow stromal cells are Lof(11-10) cells.

12. The method according to claim 11, wherein the immortalized bone marrow stromal cells are Lof(11-10) cells.

13. A method of testing the effectiveness of a gene therapy protocol against a retrovirus comprising the steps of:

i) irradiating a first and a second mouse with a lethal or non-lethal dose of radiation;

ii) administering to the irradiated mice immortalized human or non-human primate bone marrow stromal cells which express cytokines supporting growth of CD34+ stem cells;

iii) introducing a proposed therapeutic gene segment into a first group of human CD34+ stem cells;

iv) purifying the cells from the first group of CD34+ stem cells expressing the gene segment;

v) introducing a control gene segment into a second group of human CD34+ stem cells;

vi) administering the purified cells from step (iv) to the first irradiated mouse and administering the cells from step (v) to the second irradiated mouse;

vii) challenging the first and the second irradiated mice with the retrovirus; and

viii) comparing the survival of the human CD34+ stem cells from the first mouse and the second mouse.

14. The method according to claim 13, wherein the retrovirus is HIV.

15. The method according to claim 13, wherein the first and the second mouse are SCID mice.

16. An animal model for the study of human hematopoiesis comprising a rodent into which human or non-human primate CD34+ stem cells have been engrafted in the present of immortalized human or non-human primate bone marrow stromal cells.

17. The animal model according to claim 16, wherein the rodent is a mouse.

18. The animal model according to claim 17, wherein the mouse is immunosuppressed.

WHAT IS CLAIMED IS:

1. A method of engrafting human or non-human primate stem cells in a mouse comprising the steps of:

i) irradiating the mouse with a lethal or non-lethal dose of radiation;

ii) administering to the irradiated mouse immortalized human or non-human primate bone marrow stromal cells; and

iii) administering to the irradiated mouse purified human or non-human primate stem cells.

2. The method according to claim 1, wherein the immortalized bone marrow stromal cells and the purified stem cells are administered simultaneously or sequentially.

3. The method according to claim 2, comprising first administering said immortalized bone marrow stromal cells, followed by administering said purified stem cells.

4. The method according to claim 3, wherein said purified stem cells are administered about 10 days after the immortalized bone marrow stromal cells are administered.

5. The method according to claim 4, wherein said purified stem cells are administered 2-7 days after the immortalized bone marrow stromal cells are administered.

6. The method according to claim 1, wherein the immortalized bone marrow stromal cells are administered by intraperitoneal injection.

7. The method according to claim 1, wherein the purified stem cells are administered by intravenous injection.

8. The method according to claim 1, wherein the purified stem cells are purified CD34+ stem cells.

9. The method according to claim 1, wherein the mouse is immunosuppressed.

10. The method according to claim 9, wherein the mouse is a SCID mouse.

11. The method according to claim 1, wherein the immortalized bone marrow stromal cells are transfected with SV40 large T-antigen.

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GCTAAATTCC GACAAAACGA ACAGGTCG 28

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CGATTGCTTT AGAATGTGGT TTGGACTTGA TC 32

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CCTCCCTGTC TCTTTTATGC TTGTGATC 28

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CCTGGTGTTG ATGCAATGTA CTGCAAAC 28

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CACAATTTGA TGACTAAGAT TAACAAACAC 30

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GTCGACCAGT TGTTCCTTTG AGGTCCG 27

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GAGGACACCA TGCGCTTCAG AGATAACACC 30

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GGAACCTGCA GGTTTTTGGT GCAGGAGGAC 30

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CCAAAAGACT ACATGATAAC CCTCAAATAT G 31

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CCTTCTATCA TGTAAGCTAT GGCCCACTCC 30

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGTCCATATG AACTGAAAGC TCTCCACCTC 30

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GAAAAAGATG GATGCTTCCA ATCTGG 26

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GAACTGAGAG TGATTGAGAG TGGACCACAC 30

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CGCCTGGAGC TGTACAAGCA GGGCCTGCGG 30

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CTGCAGGCCC TGGAAGGGAT CTCCCCCGAG 30

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATGACTTCCA AGCTGGCCGT GCT 23

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CTCTTCAAAA ACTTCTCCCG ACTCT 25

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATGTGGCTGC AGAGCCTGCT GC 22

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGGAAACTGA CGACCCTCGG TC 22

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GAGTGTGCCA CCTACAAGCT GTGCC 25

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CGGGGACGGG ACGTCGGGTG GGTCC 25

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GAGGAGGTGT CGGAGTACTG TAGCCACATG 30

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTACTGTTCC GGACGCAGGC TTGAAAGATA C 31

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

ATGGTCCCCT CGGCTGGACA G 21

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

ACGGTCGGTC TTCTTCGTCC GG 22

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CCACTGTTTG TGCTGGATCG CAGCGCTGCC 30

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GTATCTGTTT GAACACTTAT ATCACCTACT G 31